Biological characters and rDNA ITS sequences of pathogen of poplar leaf blight

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Abstract: DNA was extracted from the strain of pathogen of poplar leaf blight using a modified CTAB method. ITS sequence (601bp) was initially amplified from the pathogen by using the universal primers ITS1 and ITS4 (registered No. DQ011257). Comparing to the nucleotide sequences acquired from GenBank database, the strain is clustered into the homogeneity with *Alternaria alternate* (AY787684) and *Alternaria alternate* (AY354228), with a homology of 98%, thus the strain was checked as *Alternaria alternata* (Fr.) Keissler. The optimal conditions for conidia germination and mycelium growth of the pathogen were tested. The optimal temperature for conidia germinating and mycelium growth is 25ºC, and the optimal pH value is 6. Mycelium grows rather slowly at 10 ºC and 30 ºC and growth stops at above 35 ºC. Among the six culture mediums tested, PDA + poplar leaf juice medium is most favorable for mycelium growth.
Keywords: Pathogen; Popular leaf blight; *Alternaria alternate*; rDNA ITS sequences; Biological characteristics
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Introduction

Poplar leaf blight is one of the important diseases in northeast China, which is more epidemic in nursery at present (Xu *et al.* 1986), and it has already caused serious economic loss. The traditional classification and identification on fungi mostly depended on the characters of morphology, physiology, histology, and anatomy, and classification in genus was often disputed and easy to be confusable. In recent years, the techniques of identifying and testing pathogen and diagnosing diseases by using PCR to amplifying rDNA internal transcribed spacer (ITS) region of pathogen have been rapidly developed. rDNA ITS regions of fungi have either conservative or specific sequences on the level of family, genus and species, and have a rapid evolution speed, so they have already been applied to bio-intraspecific variation and molecular genealogy on the relationship of interspecies and inter-genus (Zhao 2004, Wang *et al*. 2002).

However, little is known about bio-characters of the pathogen of poplar leaf blight. This study detected the pathogen of poplar leaf blight and analyzing phylogeny by using rDNA ITS sequences, and the morphological characters of the pathogen of popular leaf blight were described. This paper can provide scientific basis for studying epidemic regularity and comprehensive prevention technology of this disease.

Materials and methods

DNA extraction

The fresh mycelium (50 mg) of the pathogen strain of popular leaf blight placed in a pre-cooled mortar were frozen in liquid nitrogen and crushed with a pestle. DNA was extracted by a modified CTAB method and stored at –20°C (Xing *et al*. 2004). Its concentration was measured by Bio Photometer (Eppendorf AG 22331 Hamburg).The strain of pathogen (*Alternaria alternata*) used in the test was come from Northeast Forestry University, Harbin, China.

Amplification of ITS region and cloning, sequencing and analyzing of its amplification products

The universal primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' were synthesized by TaKaRa Company (Dalian, China) (Xing *et al*. 2004).

DNA amplification was performed with 50-μL reaction mixture containing 40 ng of template DNA, 0.4 $U \cdot \mu L^{-1}$ *Taq* DNA polymerase (TaKaRa), 0.3 $mmol \cdot L^{-1}$ dNTPs, 0.1 $\mu mol \cdot L^{-1}$ primers of ITS1 and ITS4 in a PTC-200 DNA Thermal Cycler program for 31 cycles as follows: 30 cycles of 5 min at 94°C, 0.5 min at 94°C, 45 s at 58°C with extension of 90 s at 72°C in each subsequent cycle followed by one final extension cycle of 10 min at 72°C. PCR product was size-separated by gel electrophoresis on 1% agarose gel, photographed with a UVG Image Analysis System (USA), purified by Agarose Gel DNA Purification Kit (TaKaRa), and then linked to vectors of pGEM-T (Promega). Ligation products were transformed into electrocompetent cells of *Escherichia coli* TOP10 and the plasmids of transformants were analyzed by the universal primers of T7 and SP6, and then sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd..

Acquired ITS sequences were compared with non-redundancy nucleotides database by BLASTn to determine the homology of ITS gene sequences. Multiple sequences alignment was done by the software of BioEdit v 5.06, and phylogenetic tree was constructed by neighbor-joining method in software of MEGA (Version 2.1).

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Optimal conditions for conidia germination and mycelium growth of pathogen

Temperature:

Germination test of conidia was performed by pendent drop method (Septo-Schubert *et al.* 1999, Wang *et al.* 2004, Lou *et al.* 2002, Wang *et al.* 2000, Dong *et al.* 1994, Lv *et al.* 1999, Xiang 1991). Conidia were suspended in sterile water (pH=7), and incubated on a plate for 24 h separately at 10ºC, 15ºC, 20ºC, 25ºC, 30ºC, and 35ºC. The germination rates were observed with microscope, with three replications on each plate and temperature.

Mycelium growth was measured by growth-rate method (Septo-Schubert *et al.* 1999, Wang *et al.* 2004, Lou *et al.* 2002, Wang *et al.* 2000, Dong *et al.* 1994, Lv *et al.* 1999, Xiang 1991). A colony of pathogen strain with a diameter of 0.5 cm was cut to culture on modified PDA medium (Potato 200 g, glucose 20 g, $MgSO_4$ 1.5 g, NaH_2PO_4 3 g, agar 20 g, water 1 000 mL, pH 6) at each temperature for seven days, then measuring the diameter of colony. Every culture was repeated 3 times, with 3 replications at each temperature.

pH value

Conidia were incubated at PDA mediums with different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) at 25 ºC for 24 h. Then the germination rates of conidia were observed with microscope. A colony of pathogen strain with a diameter of 0.5 cm was cultured on modified PDA medium with different pH at 25 ºC for seven days, then measuring the diameter of colony.

Nourishing solutions

The conidia suspension solutions, including PD culture medium, 2% glucose solution and sterile water, respectively, were cultured at 25 ºC for 24 h. The germinating rates of conidia were measured by microscope.

Culture medium

Six mediums were selected: modified PDA, PDA + poplar leaf juice (30-g poplar leaf juice in modified PDA medium), PS (potato 200 g, sucrose 20 g, agar 20 g, water 1 000 mL, pH 6), Czapaek (sucrose 30 g, $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, agar 20 g, water 1 000 mL, pH 6), PCA (potato 100 g, carrot 100 g, agar 20 g, water 1 000 mL, pH 6), and WF-P (sucrose 20 g, peptone 5 g, $Ca(NO_3)_2$ 0.5 g, Na_2HPO_4 2 g, $FeSO_4$ 0.5 g, agar 20 g, water 1 000 mL, pH 6).

A colony of pathogen strain with a diameter of 0.5 cm was cut to culture on different mediums at 25ºC for seven days, then measuring colony diameter by cross intersect method. Every culture was repeated 5 times.

Results

Morphology and cultural characters of pathogen

The pathogen mycelium grows faster. A colony with a diameter of 0.5 cm cultured on modified PDA plate medium (Diameter=9 cm) can expand to full plate in seven days. On modified PDA, the colony is gray-white, villiform, producing conidia in 8–10 days. Conidiophores are erect, ramose, green-brown, producing 10 or so catenate conidia. The conidia have beak, catenate, obclavate; smooth surface and deep brown; producing several transverse septa at first, then forming mediastinum (Fig. 1 and Fig. 2).

Fig. 1 Symptom of poplar leaf blight

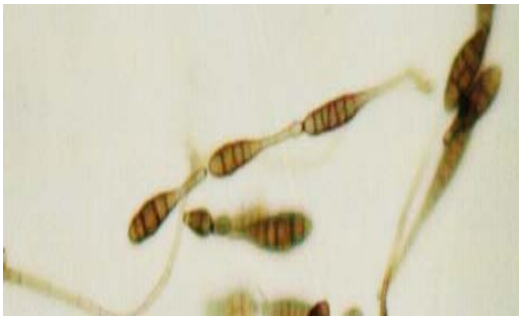

Fig. 2 Morpha of pathogen conidia

Amplification and analysis of ITS sequence

The ITS region of the nuclear ribosomal DNA from pathogen (DQ011257) comprises 601 bp (Fig.3 and Fig.4).

1 GTCAGCGGGT ATCCCTACCT GATCCGAGGT CAAAAGTTGA AAAAAAGGCT TAATGGATGC
61 TAGACCTTTG CTGATAGAGA GTGCGACTTG TGCTGCGCTC CGAAACCAGT AGGCCGGCTG
121 CCAATTACTT TAAGGCGAGT CTCCAGCAAA GCTAGAGACA AGACGCCCAA CACCAAGCAA
181 AGCTTGAGGG TACAAATGAC GCTCGAACAG GCATGCCCTT TGGAATACCA AAGGGCGCAA
241 TGTGCGTTCA AAGATTCGAT GATTCACTGA ATTCTGCAAT TCACACTACT TATCGCATTT
301 CGCTGCGTTC TTCATCGATG CCAGAACCAA GAGATCCGTT GTTGAAAGTT GTAATTATTA
361 ATTTGTTACT GACGCTGATT GCAATTACAA AAGGTTTATG TTTGTCCTAG TGGTGGGCGA
421 ACCCACCAAG GAAACAAGAA GTACGCAAAA GACAAGGGTG AATAATTCAG CAAGGCTGTA
481 ACCCCGAGAG GTTCCAGCCC TCCTTTCATA TTTGTGTAAT GATCCCTCCT CAAGTTTCTT
541 TCCTCGGAAC TTTTTTCATC AAAAAAACCA ATTTCCCTAT TTCTATGATA GCGTTTGTTT
601 T

Fig.3 ITS sequence of pathogen (*Alternaria alternate*)

Notes: 1–36= 18S gene (in bold) partial sequence, 32–194=ITS1 complete sequence, 195–353=5.8S gene (in bold) complete sequence, 354–511=ITS 2 complete sequence, 512–601=28S gene (in bold). the ITS1 sequences of pathogen was amplified using the universal primers.

Compared with the nucleotide sequences acquired from GenBank database, ITS sequences from *Alternaria alternata* was quite similar to, especially to the known genus from *Alternaria* spp. To study the systematic relationship of development, the sequences of ITS1, ITS1+5.8S, 5.8S, 5.8S+ITS2, ITS2, ITS1+ITS2 were separately used to construct phylogenetic tree. Result showed that phylogenetic tree constructed by ITS1 was almost the same with that constructed by ITS1+5.8S, 5.8S+ITS2, ITS2, ITS1+ITS2 and significantly different from that constructed by 5.8S. Thus ITS1 sequences were selected and the neighbor-joining method was used to construct phylogenetic tree. (Fig.5)

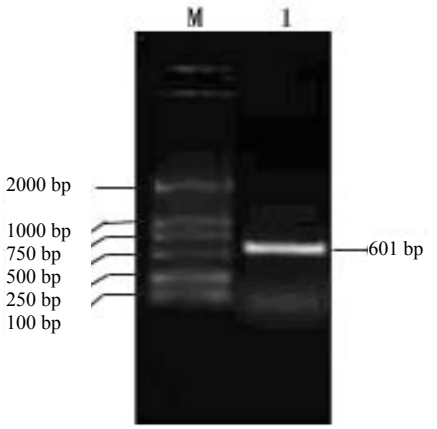

Fig.4 PCR products of rDNA ITS of pathogen. M: ladder

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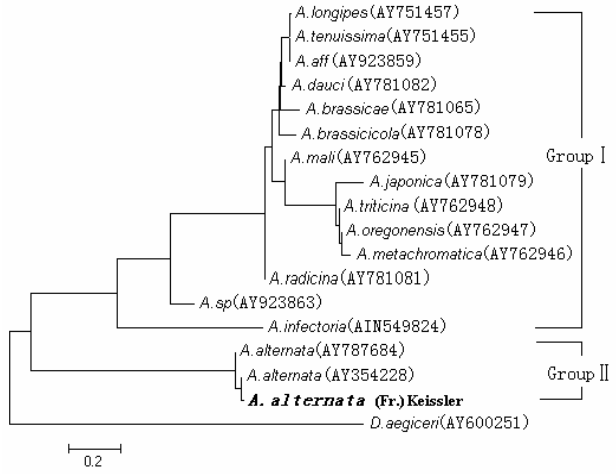

Fig.5 UPGMA phylogenetic tree based on the rDNA ITS1 sequences of *Alternaria* and relating species (*Dothiorella aegiceri* of aliens)

The results of cluster analysis (Fig.5) indicated that there was a certain extent genetic diversity in *Alternaria*. This genus can be divided into two groups (species). Group Ⅰ is *A. alternata*, including *A. alternata* (AY787684) and *A. alternate* (AY354228). Group Ⅱ includes other species except for *A. alternate*. The similarity among *A. alternata*, *A. alternata* (AY787684) and *A. alternata* (AY354228) is the highest, up to 98%, so they were the same specie. While the similarity between *A. alternata* and clade Ⅱ including *A.* sp. (AY923863) and *A. triticina* (AY762948) was the least, only 90%, so they belonged to different species in the same genus.

Effect of temperature on conidia germination

Conidia of *A. alternata* can germinate at 10–35ºC, and the appropriate temperature is 15–30ºC. The optimal germination temperature is 25 ºC, with 70.9% of germination rate in 24 h (Table 1).

Table 1. Conidia germination rates in 24 h at different temperature

Temperature (℃)	Number of conidia	Number of germinated conidia	Germination rate (%)
10	235	36	15.3
15	169	65	38.5
20	187	105	56.1
25	268	190	70.9
30	214	108	63.4
35	196	29	14.8

Effect of pH on conidia germination

Conidia are not able to germinate at pH value more than 10 (Table 2), and it can germinate at a pH of 4-9. The appropriate pH is from 5 to 7. The optimal pH is 6, with 71.2% germination rate in 24 h.

Table 2. Conidia germination rates in 24 h at different pH values

pH	Number of conidia	Number of germinated conidia	Germination rate (%)
4	256	22	8.6
5	198	108	54.5
6	233	166	71.2
7	189	112	59.3
8	253	59	23.3
9	167	9	5.4
10	210	0	0

Effect of nourishing solution on conidia germination

The results show that different kinds of nourishing solutions have little effect on conidia germination. The maximal germination rate is 78.3% on PD culture medium whose nutrition was the most abundance, and the minimum is 68.7% in aseptic water whose nutrition was correspondingly smallness (Table 3).

Table 3. Conidia germination in 24 h at different nourishing solutions

Nourishing solution	Number of conidia	Number of germinated conidia	Germination rate (%)
PD culture medium	263	206	78.3%
2% glucose solution	215	154	71.6%
Aseptic water	227	156	68.7%

Effect of temperature on mycelium growth

Effect of temperature on growth of pathogen mycelia was significant. Mycelia can grow at 10–30 ºC, and the optimal temperature is 25 ºC. It grows rather slowly at 10 ºC and 30 ºC, and growth stops at 35 ºC or more (Table 4).

Effect of pH on mycelium growth

Effect of pH on the growth of pathogen mycelia was notable. The mycelia can grow at the pH of 4-9. The better pH is 5-7, and the best is 6 (Table 4).

Table 4. The changes of colony diameters at different conditions in 7 days

Conditions		Colony diameter (cm)
Temperature (℃)	10	1.8
	15	3.4
	20	5.8
	25	8.2
	30	2.6
	35	0
pH	4	5.2
	5	7.5
	6	8.3
	7	7.4
	8	4.8
	9	1.6
	10	0
Medium	PDA	8.0
	PDA+juice of the polar	8.1
	PS	7.8
	Czapaek	5.7
	PCA	8.3
	WF-P	4.9

Effect of different culture mediums on mycelium growth

The mycelium can grow on modified PDA, PDA+ juice of the polar, PS, Czapaek, PCA and WF-P. The colony diameter on PCA is the biggest, but the growth trend is not as strong as that on PDA, PDA+ juice of the polar, and PS. It grows best on medium of PDA+ juice of the polar, the worst on WF-P medium.

Discussion and conclusion

ITS sequence was amplified successfully from pathogen of poplar leaf blight with the universal primers ITS1 and ITS4 firstly. Length of the ITS sequence is 601 bp: 1–31 bp is partial sequence of 18S rRNA, 32–194 bp is ITS1, 195-353 bp is 5.8S rRNA, 354–511 bp is ITS2, 512-601 bp is partial sequence of 28S rRNA (GenBank No. DQ011257). This is closer to *Alternaria alternata* (AY787684) and *Alternaria alternata* (AY354228) and the concordance ratio is 98%. Therefore the pathogen strains has been checked as *Alternaria alternata* (Fr.) Keissler.. It belongs to Deuteromycotina, Hyphomycetes, Hyphomycetales, genus of *Alternaria*.

The sequence 5.8S is very important in the division of genus as rather conservative "the epibiotic biological-clock fossil". Our study shows that the conservation of gene sequences of *Alternaria alternata* is less than that of other species in the same genus. About 60 bases, which belong to 5.8S sequence of *Alternaria alternata,* are not homological. The results show that the evolutionary relationship of *Alternaria* is more complex, *Alternaria alternata* lies on the truck of the phylogenetic tree and is relative epibiotic, the differentiation of inter-genus species is more serious (Fig. 5).

The optimal temperature and pH for conidia germination of *Alternaria alternata* is 25ºC and 6, respectively. Nutrition is beneficial to the conidia germination. The optimal temperature and pH value for mycelium growth are 25ºC and 6, respectively. The pathogen grows best in the medium of PDA+ juice of the polar.

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